Sulfonate desulfurization in *Rhodococcus* from wheat rhizosphere communities

Achim Schmalenberger[1,3], Sarah Hodge[1], Malcolm J. Hawkesford[2], and Michael A. Kertesz[1,*]

[1]Faculty of Life Sciences, University of Manchester, Oxford Rd, Manchester, M13 9PT, United Kingdom, [2]Plant Sciences Department, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, United Kingdom, [3]Cell-Mineral Research Centre, Kroto Research Institute, University of Sheffield, Broad Lane, Sheffield, S3 7HQ, United Kingdom.

Target journal: **FEMS Microbiology Ecology**

Keywords: Broadbalk, wheat, DGGE, multiplex T-RFLP, *asfA*, sulfonate utilization, desulfonation, *Rhodococcus*, actinobacteria

Running title: Desulfonation by rhizosphere *Rhodococcus*

*Corresponding author: Michael A. Kertesz

Faculty of Life Sciences,

University of Manchester,

D1401 Michael Smith Building, Oxford Rd,

Manchester M13 9PT, England.

tel: +44-161-275 3895

fax: +44-161-275 5656

e-mail: michael.kertesz@manchester.ac.uk

Abstract

Organically bound sulfur makes up about 90% of the total sulfur in soils, with sulfonates often the dominant fraction. Desulfurization of these sulfonates by rhizobacteria involves the oxidoreductase AsfA. Here, we report that actinobacteria affiliated to the genus *Rhodococcus* were able to desulfonate arylsulfonates in wheat rhizosphere, and analyse the impact of different sulfur fertilization regimes on the actinobacterial community in wheat rhizosphere. Isolates and DNA samples were obtained from the Broadbalk long-term field wheat experiment, Rothamsted UK, which includes plots treated with inorganic fertilizer with and without sulfate, with farmyard manure, and unfertilized plots. Direct isolation of desulfonating rhizobacteria yielded *Rhodococcus* strains which grew well with a range of sulfonates and contained the *asfAB* genes. Expression of *asfA in vitro* increased >100-fold during growth of the *Rhodococcus* isolates or *Rhodococcus* sp. RHA1 in the presence of toluenesulfonate as sole sulfur source, compared to growth with sulfate. By contrast, the closely related *Rhodococcus erythropolis* and *Rhodococcus opacus* type strains had no desulfonating activity and did not contain *asfA* homologues. The actinobacterial community structure in wheat rhizospheres was influenced by the sulfur fertilization regime, as shown by denaturing electrophoresis of specific 16S rRNA gene fragments. Clone library analysis at the *asfAB* functional gene level identified nine different *asfAB* genotypes closely affiliated to the *Rhodococcus* isolates. However, *asfAB*-based multiplex RFLP/T-RFLP analysis of wheat rhizosphere communities based on these genotypes revealed no significant differences between the fertilization regimes, suggesting that the desulfonating *Rhodococcus* community does not specifically respond to changes in sulfate supply.

1. Introduction

Forty years ago, atmospheric SO_2 pollution in Britain was so high that annual rates of sulfur deposition were approximately 70kg/ha, and up to 80% of the sulfur taken up by plants originated from atmospheric sources (Zhao *et al.*, 2003). Pedospheric sulfur levels were therefore very rarely limiting for plant growth at that time, but by 1995 this had changed, and reductions in air pollution meant that a third of British agricultural land was at medium to high risk of sulfur deficiency (McGrath & Zhao, 1995). These risk levels were predicted to reach >50% by today, and indeed, sulfur deficiency has continued to be problematic in many crop-growing regions (Scherer, 2001). Plants rely on inorganic sulfate as their main sulfur source, mainly incorporating the assimilated sulfur into cysteine and methionine, but also using it in enzymatic electron transfer processes and in protection against oxidative and heavy metal stress (Meyer & Hell, 2005; Sharma & Dietz, 2006). However, sulfur in soils is mostly not free inorganic sulfate, but is organosulfur, bound to soil organic matter. The sulfur that is found in the plant material that makes up the litter layer is mainly (60-90%) in carbon-bound form (Zhao *et al.*, 1996). Chemical and spectroscopic investigations of soil sulfur in a range of soils have revealed that up to half of the sulfur pool corresponds to sulfonate-sulfur (Autry & Fitzgerald, 1990; Zhao *et al.*, 2006). While early studies suggested that the sulfate ester pool was the main source of sulfur plant growth, mineralization rates for soil sulfur correlate better with the content of sulfonate-sulfur than with sulfate-ester sulfur, suggesting that sulfonates are important for plant sulfur nutrition (Ghani *et al.*, 1992; Zhao *et al.*, 2006).

Soil sulfur cycling is largely influenced by the microbial mineralization of organosulfur compounds (Kertesz & Mirleau, 2004; Kertesz *et al.*, 2007). Previous studies of soil organosulfur metabolism have focused on the microbial sulfatase

activities which are responsible for sulfate-ester desulfurization (Freeman & Nevison, 1999; Klose *et al.*, 1999; Knauff *et al.*, 2003; Taylor *et al.*, 2002; Vong *et al.*, 2003), and very little is known about the microbial sulfonatase activity, partly because no straightforward sulfonatase assay is available. Microbial desulfurization of aliphatic and aromatic sulfonates has been best studied in *Pseudomonas putida* S-313, which can desulfurize a broad range of sulfonates (Vermeij *et al.*, 1999; Zürrer *et al.*, 1987). The aliphatic desulfurization reaction is catalysed by an enzyme complex consisting of the SsuD monooxygenase and SsuE FMN reductase, while cleavage of sulfur from aromatic structures also requires the reductase/ferredoxin couple AsfA and AsfB (Vermeij *et al.*, 1999). The AsfAB gene products have recently also been implicated in desulfurization of aromatic sulfonates in a range of beta-proteobacteria, including *Cupriavidus metallidurans, Variovorax paradoxus* and rhizosphere isolates of *Variovorax*, *Acidovorax* and *Polaromonas* (Schmalenberger & Kertesz, 2007; Schmalenberger *et al.*, 2008). Molecular analysis of barley and wheat rhizospheres revealed considerable diversity of *asfA* orthologues, most of which were closely affiliated to *asfA* sequences of beta-proteobacteria (Schmalenberger & Kertesz, 2007; Schmalenberger *et al.*, 2008). In a study of wheat rhizospheres on the Broadbalk long term experiment, it was found that the population of specific members of the *Comamonadaceae*, in particular, appeared to respond to changes in sulfur fertilization regime (Schmalenberger *et al.*, 2008). Together, these results suggest that particular groups of rhizobacteria may carry out organosulfur mineralization processes, in response to differing levels of sulfate availability.

However, organisms outside the proteobacteria have also been shown to desulfurize organosulfur compounds in the past. *Rhodococcus* species are versatile in this respect, and have been studied in detail for their ability to desulfurize coal- and

oil-derived material, in particular dibenzothiophene and dibenzothiophene derivatives (reviewed by (Kilbane, 2006)). A *Rhodococcus* species was also able to degrade linear alkyldiphenyletherdisulfonate surfactants (Schleheck *et al.*, 2003). Rhodococci are recognized to be metabolically very versatile, and active in a number of areas of biodegradation (Larkin *et al.*, 2005; van der Geize & Dijkhuizen, 2004) , but the importance of rhodococci for sulfur cycling in soils and rhizosphere has not yet been evaluated.

In this study we report several *Rhodococcus* isolates from wheat rhizospheres that can desulfurize arylsulfonates. Changes in sulfur fertilization were found to have significant effects on the actinobacterial diversity in wheat rhizospheres of the Broadbalk long term experiment (Rothamsted Research, Harpenden, UK), but the diversity of the *Rhodococcus* desulfonation genes *asfAB* did not respond significantly to sulfur fertilization, contrasting with the effect previously reported for the *Comamonadaceae*.

2. Materials and methods

2.1 Microorganisms, sampling, isolation and culture conditions.

Bacterial strains and oligonucleotides used in this study are listed in Table 1. *Rhodococcus* strains were cultivated aerobically at 25ºC in MM minimal medium (Beil *et al.*, 1995) with succinate, glucose and glycerol as carbon sources (10 mM each) and on agarose plates (14 g/l, Eurobio, France). Isolates from this study were cultivated with 24 different sulfur sources (250 µM). Wheat rhizosphere samples (Hereward cultivar) were collected from the Broadbalk long term experiment at Rothamsted, United Kingdom, (51º49'N 0º21'W). The Broadbalk experimental field is a grid divided into 20 longitudinal strips that are subjected to different fertilization

regimes, and 10 transverse sections (Rothamsted_Research, 2006). Two sections of the field (sections 1 and 9) are cropped with continuous wheat, and these were used to provide field replication of the treatments. Wheat rhizosphere samples were collected in late July 2005 from the sections 1 and 9 of strips had been fertilized with farmyard manure (plot 2.2, FYM, receiving c 250 kg/ha N per year), inorganic fertilizer, (plot 9, NPKS, receiving 192 kg/ha N per year), sulfur-free inorganic fertilizer (plot 14, NPK, receiving 192 kg/ha N per year), and an unfertilized strip (plot 3, NIL). Four samples were collected for each treatment, two in section 9 (replicates 1 and 2, approx 10 m apart), and two in section 1 (replicates 3 and 4). Wheat plants were removed as previously described (Schmalenberger *et al.*, 2008), and taken to the laboratory for further analysis.

For rhizosphere analysis, the loosely attached soil was shaken off each sample (3-6 plants), and root-associated bacteria were then extracted into sterile phosphate buffered saline (PBS: 20 ml per 3 g of root (FW)) by shaking on a Genie roto-shaker (Scientific Industries, NY) for 30 min at 4˚C. Soil bacteria able to desulfurize toluenesulfonate (TS) were identified by cultivating in most probable number (MPN) microtiter plates as described previously (Schmalenberger *et al.*, 2008). Individual TS-utilizing strains were identified for further study from the highest dilutions showing growth in MM-MPN microtiter plates. Strains were replated on MM agarose plates with TS as the sole sulfur source and single colonies were picked for subsequent analysis. Utilization of different sulfur sources by single bacterial isolates was tested during growth in microtitre plates with a range of 23 different sulfur sources including sulfonates and sulfate-esters, as described previously (Schmalenberger *et al*., 2008) and dibenzothiophene.

2.2 DNA extraction and PCR conditions

The rhizobacterial suspensions described above were subjected to direct DNA extraction using the FastDNA extraction kit for soil (QBiogene, Irvine, CA), as described previously (Schmalenberger & Kertesz, 2007). Genomic DNA from individual isolates was extracted for PCR analysis with a quick lysis protocol (Schmalenberger *et al.*, 2001).

PCR was carried out in a T1 thermocycler (Biometra, Goettingen, Germany) in a final volume of 50 µl. Amplification of *asfAB* (1.5kb *asfA* and 0.14kb *asfB*) from pure cultures was carried out with Expand polymerase/buffer (Roche, Basel, Switzerland), 1.5 mM $MgCl_2$, 5% v/v DMSO, 0.5 µM primers (asfA_RHA1 with asfBtoA), and 200 µM dNTPs, as described previously (Schmalenberger & Kertesz, 2007) but with 45 cycles. For RFLP/T-RFLP analysis, *asfAB* was amplified from environmental samples with HotMaster Taq polymerase, using the buffer and Enhancer provided by the manufacturer (Eppendorf, Germany), 5% v/v DMSO and a touch down protocol as described previously (Schmalenberger & Kertesz, 2007) with primers asfA_F_rho1 and asfBtoA.

Analysis of the actinobacterial community was carried out by 16S-PCR-DGGE, using a nested-PCR approach. The initial PCR step used primers F243Actino and 518R. The purified amplification product then served as template for a second PCR which used the universal bacterial DGGE primers GC-341F and 518R, HotMaster Taq polymerase (Eppendorf) and a touch down PCR protocol (Cunliffe & Kertesz, 2006).

2.3 Denaturing gradient gel electrophoresis (DGGE)

DGGE was carried out on 20 x 16 cm gels in a D-code electrophoresis chamber (Biorad, Hercules, CA) as described previously (Cunliffe & Kertesz, 2006). A denaturant gradient of 40 to 70% was applied and electrophoresis carried out for 17 h at 64V and 60°C. Rhizosphere community profiles were prepared with 500 ng of DNA, while samples with a defined mixture of species contained 50 ng of DNA for each species. Signals were visualized through staining for 30 min with SybrGold (Invitrogen, Carlsbad, CA). Statistical analysis of the community fingerprints was carried out by UPGMA cluster analysis (Phoretix).

For sequencing of selected DGGE bands, the bands were excised from the gel, incubated in dH_2O for 24 h at 4°C, and then homogenized in 0.5 ml of dH_2O. After 100-fold dilution, these templates were reamplified using identical primers but without the GC-clamp. Single signals were sequenced directly as described previously (Cunliffe & Kertesz, 2006) but when reamplification yielded multiple products the original PCR products were cloned. The position of signals from individual sequenced clones was then compared with the community profiles by a second DGGE.

2.4 Cloning of asfAB PCR products and genotyping

Primers asfA_F_rho1 and asfBtoA (Table 1) were used to amplify fragments of *asfAB* from wheat rhizosphere extracts obtained from each of the four treatments studied. PCR products were purified with the Qiagen PCR purification kit, ligated into the pGEM-T easy vector (Promega) and transformed into *E. coli* DH5α. Recombinant plasmids containing an insert of the correct size were then reamplified with the same primers for RFLP analysis. RFLP was carried out with 80 clones in total (NIL:20, NPK:20, NPKS:20, FYM:20), as described previously (Schmalenberger *et al.*, 2008). Clones with a similar restriction pattern were classified as a single genotype.

2.5 Restriction fragment length polymorphism (RFLP) and terminal restriction fragment length polymorphism (T-RFLP) analysis

Primer asfBtoA was modified with the fluorescent label HEX and used to amplify fragments of Rhodococcus like *asfAB* from wheat rhizosphere extracts as described above. The amplification products were purified (Qiagen PCR purification kit, Germany) and 200 ng DNA was digested with 10 U of *AluI* and *Rsa*I (Fermentas) at 37°C for 12 h. Samples were run on a 20 x 20 cm acrylamide electrophoresis gel, and RFLP and TR-F signals were identified using a Typhoon scanner (multiplex RFLP/T-RFLP) as described previously (Schmalenberger *et al.*, 2007). Statistical analysis of the binary data set was carried out by UPGMA cluster analysis, principal component analysis (PCA) and detrended correspondence analysis (DCA) using the software packages Phoretix, Canoco and Decorana.

2.6 Gene expression analysis

Expression of *asfA* in *Rhodococcus* strains P14D10, P15D9 and RHA1 was measured by quantitative RT-PCR, using the primers asfA_rho_QF and asfA_rho_QR (Table 1). Standards (10^1 to 10^8 molecules per reaction) were generated from specific PCR products, and cDNA was generated by reverse transcription of 100ng of total RNA using RevertAid reverse transcriptase (Fermentas, Burlington, Canada), following the manufacturer's instructions. Quantitative PCR was then performed in a Roche Lightcycler 2 (Roche), using 20 µl glass capillaries (Roche), containing 5 µl DyNAmo capillary SYBR Green qPCR master mix (Finnzymes, Espoo, Finland), 0.3 pmol of each primer and 1 µl of cDNA (equal to 5ng total RNA) in a total volume of 10 µl. PCR conditions were 95°C for 10min (hot start), followed by 40 cycles of 15s 95°C, 15s at 55°C and 20s at 72°C. A melting curve was carried out from 55 to 95°C at 0.1°C/s.

2.7 DNA sequence analysis

Different genotypes of *asfAB* were sequenced in order to obtain the sequence information of *asfA* orthologues. Sequence fragments were truncated to obtain just the *asfA* fragment and were imported into an *asfA* database generated previously (Schmalenberger & Kertesz, 2007; Schmalenberger *et al.*, 2008), using the ARB software package (www.arb-home.de). Trees for the encoded peptide sequences (AsfA) were calculated with the Maximum Likelihood (Dayhoff model) method.

16S rRNA gene sequences from isolates obtained in this study were imported into the 2004 SSU database of ARB, together with closely affiliated sequences from Genbank identified using BLAST (Altschul *et al.*, 1990) and FASTA3 (Pearson & Lipman, 1988). The sequences were aligned, and the sequences with the highest similarity were identified. Affiliation of sequences from DGGE bands were identified using BLAST and FASTA3.

2.8 Analytical methods

Reversed-phase high performance liquid chromatography (HPLC) was carried out on a Dionex system using a C18-Hypersil column (4mm x 25 cm). A methanol gradient in 10 mM potassium phosphate buffer was applied, and eluted compounds were detected at 200 and 220 nm, as described previously (Vermeij *et al.*, 1999).

2.9 Nucleotide sequence accession numbers

Isolates were identified by amplification and sequencing of the 16S rRNA genes with primers 27f and 1492r. Nucleotide accession numbers are AM942743 and AM942744.

Fragments of *asfA* from isolates and *R. erythropolis* IGTS8 have accession numbers AM94180 to AM94182 and from molecular isolates have the accession numbers AM94183 to AM94291.

3. Results

3.1 Identification of desulfonating Rhodococcus strains in wheat rhizospheres.

The dominant bacteria capable of desulfurizing arylsulfonates in wheat and barley rhizospheres have been shown to belong to the *Comamonadaceae* family, related to *Variovorax* and *Polaromonas* (Schmalenberger & Kertesz, 2007; Schmalenberger *et al.*, 2008). The isolates studied all contained related *asfAB* genes (Schmalenberger *et al.*, 2008) and released *p*-cresol as desulfonation product during *in vitro* growth with toluenesulfonate (TS) as sulfur source, which served as a model for soil sulfonates (Schmalenberger *et al.*, 2008). In order to test whether other rhizosphere bacteria catalyse desulfonation independently of AsfA, we isolated further TS-desulfonating strains from wheat rhizospheres obtained from the Broadbalk long-term experiment. Two desulfonating strains isolated from the FYM plot appeared morphologically distinct from the comamonads studied previously and were studied further. No cresol was found in the culture supernatants of these strains after growth of these strains with TS *in vitro* (HPLC-analysis), and PCR analysis with the *asfA*-specific primers developed earlier (asfAF2 and asfBtoA (Schmalenberger & Kertesz, 2007)) suggested that they did not contain an *asfA* gene, or that this was divergent from known *asfA* genes. The 16S rRNA gene sequences of these strains showed that both belonged to the genus *Rhodococcus,* with 99.9% (P15D9) and 100% (P14D10) identity to *Rhodococcus erythropolis*. The strains were not able to grow with 5 mM cresol as a sole carbon source, though they did co-metabolize smaller amounts of cresol during growth with other carbon sources, both in the presence and absence of TS as sulfur source. Comparison with culture collection strains of *Rhodococcus* revealed that the ability to desulfurize TS is also present in other members of this genus – although *R.*

erythropolis NCIMB11148 and *Rhodococcus opacus* DSM8531 showed no growth with TS as sole sulfur source, *Rhodococcus* sp. RHA1 and *R. erythropolis* IGTS8 showed significant growth in minimal medium with TS as sole sulfur source.

The ability to grow with a range of different sulfur sources was tested in microtitre plates for *Rhodococcus* isolates P14D10 and P15D9. Isolate P14D10 grew well on 21 of 24 sulfur sources tested, including aryl- and alkylsulfonates, sulfate-esters and amino acids, though it displayed no growth with sodium dodecylsulfate, potassium thiocyanate or dibenzothiophene. Isolate P15D9 grew well on 23 tested sulfur sources but more weakly with sodium dodecylsulfate or potassium thiocyanate and was unable to desulfurize dibenzothiophene. (Table 2).

3.2 asfAB sequences from Rhodococcus isolates

Analysis of the genome sequence of *Rhodococcus* sp. RHA1 (McLeod *et al.*, 2006) revealed the presence of genes encoding two AsfA orthologues (RHA1_ro01640 and RHA1_ro01604), which displayed 62% and 55% identity respectively to the *V. paradoxus* AsfA protein. Both these genes were associated with a putative *asfB* orthologue. The *Rhodococcus* sp. RHA1 genome also contains an extensive *ssu* gene cluster (RHA1_ro07045–RHA1_ro07053), which encodes putative homologues of two SsuD sulfonatases, a desulfinase and a transport system, as well as a divergent IclR-family transcriptional regulator. Further attempts were therefore made to amplify *asfA* from strains P14D10 and P15D9 using modified PCR protocols with up to 45 cycles, and a primer derived from the *Rhodococcus* RHA1 *asfA* sequence (RHA1_ro01640). Partial gene sequences were obtained from both isolates – the encoded protein showed 77.9% and 77.3% identity to the AsfA sequence from strain RHA1, confirming that these genes are conserved. Using these sequences a consensus

primer was designed for fingerprint analysis of environmental *Rhodococcus asfA* (asfA_F_rho_1). We also designed more general primers that amplifed a broader range of actinobacterial *asfA* genes (e.g. *Nocardia farcinica*), but unfortunately they also amplified *asfA* from the unrelated *Cupriavidus metallidurans* and were therefore not used in this study.

3.3 Regulation of asfA expression in Rhodococcus.

To test the dependence of *Rhodococcus asfA* expression on sulfur supply, *Rhodococcus* spp. P14D10, P15D9 and RHA1, were cultivated in minimal medium with sulfate (0.25 mM), toluenesulfonate (0.25 mM) or both sulfur sources (0.25 mM each). Expression of the *asfA* homologues during the exponential growth phase was evaluated by two step quantitative RT-PCR. In all three organisms the expression of *asfA* was more than 100-fold higher in the presence of toluenesulfonate as sole sulfur source than during growth in the presence of sulfate alone (Table 3). In strain P14D10 the presence of sulfate effectively repressed *asfA* expression during growth with the two sulfur sources together, but in strains P15D9 and RHA1 expression levels were significantly higher in the presence of sulfate and toluenesulfonate than with sulfate alone (Table 3). This difference in *asfA* expression pattern reflects the patterns seen in *P. putida* and *V. paradoxus*, respectively (Schmalenberger & Kertesz, 2007), suggesting different modes of regulation in the two isolates.

3.4 Actinobacterial rhizosphere community structures change with the type of fertilization applied.

Differences in the rhizobacterial communities on field plots subjected to different fertilization regimes were characterised in a cultivation-independent manner using

denaturing gradient gel electrophoresis (DGGE). Profiling of partial actinobacterial 16S rRNA genes using specific primers (Heuer *et al.*, 1997) yielded complex community fingerprints with approximately 50 distinguishable signals (Fig. 1a). Visual comparison of the profiles revealed a clear separation between the unfertilized control, the farmyard manure application and the plots subjected to inorganic fertilizer treatment. This was confirmed by cluster analysis with Phoretix UPGMA software, which identified a separation of all four treatments including the NPK and NPKS treatments, except that samples NPKS1 clustered with NPK3 and NPK4 profiles (Fig. 1b). A pairwise structuring of the results was evident, with replicate profiles from section 9 (replicates 1, 2) being highly similar, as were replicates from section 1 (replicates 3, 4). These sections are approximately 300 m apart on the Broadbalk field, and the field heterogeneity effects observed were also seen previously at the beta-proteobacterial level (Schmalenberger *et al.*, 2008).

Bands characteristic for all profiles or for a specific treatment were sequenced either directly or after cloning. Direct sequencing identified prominent genera in the community, including *Kitasatospora*, *Arthrobacter*, *Streptomyces*, *Promicromonospora*, *Cellulomonas* and an *Aeromicrobium*-related band (Fig. 1a). The cloning strategy led to identification of signals affiliated to *Rhodococcus* in NPK and NPKS profiles, *Aeromicrobium* in NPK1 and 2, *Actinobispora* in NIL and *Saccharomonospora* in FYM (Fig. 1a). Furthermore, a band related to *Nocardia* was present in all profiles, at different abundances (Fig. 1a). The results confirm that the class-specific primers were selecting for actinobacteria effectively, and expand the range of actinobacterial species known to colonize wheat roots.

3.5 Rhodococcus asfAB gene diversity in wheat rhizospheres from different fertilization regimes.

The response of *asfAB* gene diversity to changes in sulfur fertilization was examined to determine whether changes in sulfur supply to the plants selected for specific *Rhodococcus* genotypes, as seen before for the *Comamonadaceae* (Schmalenberger *et al.*, 2008). Gene libraries of *asfAB* (containing 0.7kb of *asfA* and 0.14kb of *asfB*) were constructed from samples from each fertilization regime, using primers asfA_F_rho1 and asfBtoA. A total of 80 individual clones were screened by RFLP analysis and nine distinct *asfAB* genotypes were discovered (coverage: 56%), yielding eight genotypes with unique *asfA* sequences. After translation, the obtained sequences were combined with the AsfA sequences of strains P14D10, P15D9, IGTS8 and RHA1, *Nocardia farcinica* and several proteobacteria such as *Variovorax paradoxus* (Fig. 2) and a maximum likelihood tree was generated. The sequences obtained in this study fell into a single clade with an identity of 96 to 99% which also included *R. erythropolis* IGTS8, while AsfA of *Rhodococcus* sp. RHA1 and *N. farcinica* clustered only remotely alongide this clade. The identity of the AsfA sequences from strain IGTS8 and the *Rhodococcus* isolates and clones to those of *Rhodococcus* sp. RHA1 and *N. farcinica* was 74% and 70% respectively.

Community *asfAB* profiles were obtained for all four samples from each fertilization regime, using a multiplex RFLP/T-RFLP approach. T-RFs were detected using a HEX labelled reverse primer and RFLP fragments were visualised after SybrGold staining. In total, 10 distinct T-RF signals were identified with three of them dominating the profiles. Cluster analysis using UPGMA, PCA and DCA did not reveal any treatment-specific clustering of the signals in either the RFLP profiles or T-RFLP profiles (not shown). However, an integrated DCA analysis of RFLP and T-RF

signals identified a moderate separation of NPK1-2 and NPK 3-4 treatments (Fig. 3) but no separation of the NIL treatment. In the Broadbalk experiment the fertilization regime therefore appears have no strong selection effect for *Rhodococcus* strains with specific *asfA* genotypes, in contrast to the situation observed for *Comamonadaceae* (Schmalenberger *et al.*, 2008).

4. Discussion

Carbon-bound sulfonate-sulfur makes up a considerable proportion of the sulfur present in agricultural soils, and its mobilization for plant assimilation is largely dependent on microbial reactions in the soil and rhizosphere. Recently, it was reported that members of the *Comamonadaceae* play an important role in this process, and that comamonad *asfA* gene diversity responds to changes in sulfur fertilization (Schmalenberger *et al.*, 2008). Here, we describe the first desulfonating rhizosphere isolates of *Rhodococcus*, and report that although the overall actinobacterial community in wheat rhizospheres changed with four different sulfur fertilization regimes, the *Rhodococcus asfAB* diversity showed no significant variation, suggesting that *Rhodococcus* species play a less significant role in sulfonate mineralization in the Broadbalk wheat rhizospheres than members of the *Comamonadaceae*.

The link between *Rhodococcus* and desulfurization is a well-studied one, but has concentrated on the removal of dibenzothiophene derivatives from fuels, with a view to developing biodesulfurization processes (Gupta *et al.*, 2005). Growth of *Rhodococcus* isolates with dibenzothiophene as sulfur source involves its oxidation to the sulfone derivative, cleavage to yield a sulfinate, and then removal of the sulfinate group by the DszB desulfinase (Oldfield *et al.*, 1997). The mechanism is widespread in actinobacteria (Gilbert *et al.*, 1998; Hirasawa *et al.*, 2001; Maghsoudi *et al.*, 2000;

Maghsoudi *et al.*, 2001), but has been best studied in *R. erythropolis* IGTS8. Early work suggested that a sulfonated intermediate rather than a sulfinate was involved in the desulfurization (Denome *et al.*, 1993; Gallagher *et al.*, 1993), but sulfonate cleavage by *Rhodococcus* species has only been observed in an isolate that desulfurized a mixture of disulfodiphenylether carboxylates (Schleheck *et al.*, 2003). Here, we have identified several *Rhodococcus* strains that cleave sulfonates to provide sulfur for growth, including *Rhodoccus* sp. RHA1, for which the genome sequence (McLeod *et al.*, 2006) contains the conserved *asfA* and *ssuD* genes, which are involved in the desulfonation process in other genera (Kertesz & Wietek, 2001). AsfA expression in the strains studied was regulated by sulfur supply (Table 3), and a similar desulfonation mechanism may be active in *Rhodococcus* as is found in *Pseudomonas* or *Variovorax* species (Schmalenberger & Kertesz, 2007), despite their phylogenetic divergence. In particular, it seems likely that desulfonation in the *Rhodococcus* species studied is indeed mediated by AsfA and probably leads to the production of the corresponding phenol as desulfurization product – this was not apparent here because unlike *Pseudomonas* or *Variovorax* the cells were able to metabolize the cresol product from TS during growth. The presence of desulfonating rhodococci in wheat rhizospheres suggests that the ability to desulfurize aromatic sulfonates and compounds such as dibenzothiophene may be widespread, and not only found in hydrocarbon-contaminated soils.

The rhizosphere constitutes a highly favourable environmental niche for bacterial growth, with up to a hundred times more activity than in bulk soil (Curl & Truelove, 1986) but it also contains lower concentrations of nutrients like nitrate, phosphate and sulfate since plants and microorganisms compete for these elements (Hinsinger *et al.*, 2005). This competition can be enhanced for experimental purposes

by manipulating the fertilizer regime, and we have used the Broadbalk long-term wheat experiment to evaluate sulfate-depletion effects, since it has included a sulfur free fertilization treatment (NPK) since 2001. The effectiveness of this treatment was confirmed by measurements of sulfate and total sulfur in wheat rhizospheres from Broadbalk in 2005, after four years of treatment. At that time, total sulfur concentrations in the sulfate-depleted plot (NPK) and the control plot (NIL) were comparable, and levels of inorganic sulfate in the NPK plot were reduced by 50% compared to the sulfate fertilized plots (NPKS) (Schmalenberger *et al.*, 2008), although grain yields in NPK and NPKS plots were not significantly different (Rothamsted_Research, 2008). Wheat rhizospheres have been shown to harbour a diverse range of microorganisms. A cultivation-dependent analysis identified 28 different genera, with the cultivable rhizosphere bacterial community in a modern cultivar dominated by *Pseudomonas* and *Arthrobacter* species (Germida & Siciliano, 2001). The community structure is expected to vary with cultivar and soil type, though a number of microorganisms have been consistently isolated from wheat rhizospheres in soils from around the world (Drinkwater & Snapp, 2007). For the Broadbalk rhizospheres the dominant members of the actinomycete community were *Arthrobacter* and *Streptomyces* (Fig. 1a), though the actinobacterial community structures varied in response to the fertilization regime (Fig. 1a). Other studies have also shown that rhizobacterial community structure reacts to changes in land use, with significant effects seen for such factors as fertilization and plant species (Clegg *et al.*, 2003; Enwall *et al.*, 2007; Innerebner *et al.*, 2006; Schmalenberger & Tebbe, 2002; Seghers *et al.*, 2005; Seghers *et al.*, 2003; Stark *et al.*, 2007). Actinobacteria are important players in the grassland environment, and respond to changes in grassland management regimes (Clegg *et al.*, 2003), and they are also affected by addition of

organic and mineral fertilizers to soil (Stark *et al.*, 2007). In the Broadbalk rhizospheres the general effect of fertilization (inorganic or manure application) on the actinobacterial community was different from the response of the beta-proteobacterial rhizosphere communities (Schmalenberger *et al.*, 2008). Here, the most substantial differences occurred between the unfertilized (NIL) and the fertilized plots (NPK, NPKS, FYM). The distinct levels of sulfur in the fertilizers had a less dramatic effect on the actinobacterial community, suggesting that increased levels of nitrogen and phosphorus were the strongest drivers in the differentiation of the communities. Levels of organic matter and sulfur had a lower impact on the actinobacterial community.

It is important to note that the study reported here was carried out in a long-term monoculture experiment, and that the communities observed in other agricultural regimes may differ. Long-term cultivation with a single crop enriches for rhizosphere inhabitants that are specifically adapted to the rhizosphere of that crop (Landa *et al.*, 2006; Mazzola *et al.*, 2004), and specific cultivars play an important role in determining the community that develops (Mazzola *et al.*, 2004), with the effect increasing as the duration of monoculture increases (Gardener & Weller, 2001). Several long-term field studies showed that management regimes have a strong impact on soil quality and soil microbial populations (Böhme *et al.*, 2005; Hartmann & Widmer, 2006; Marschner *et al.*, 2003), and we are currently expanding our studies to examine the desulfonation community in the rhizospheres of other crops and in natural grassland.

At the outset of this study we anticipated that wheat rhizospheres with differing sulfur content would select not only for beta-proteobacterial species (Schmalenberger *et al.*, 2008) but also for actinobacteria with specific functions in

sulfur mobilization. Indeed we observed an effect of sulfur fertilization on the overall actinobacterial community by 16S rRNA gene fingerprint analysis (Fig. 1). However, *Rhodococcus asfA* diversity did not respond significantly to differences between the fertilization regimes. Only a combined analysis of RFLP and T-RF data revealed a moderate separation of NPK from the other treatments. No specific signal in the *asfAB* based analysis could be linked to the levels of sulfate in the rhizosphere soils, suggesting that desulfonating rhodococci occur independently of the sulfate level in the wheat rhizosphere. This finding contrasts with the changes in diversity of desulfonating *Comamonadaceae* seen in the same environment (Schmalenberger *et al.*, 2008). Microorganisms that respond to low sulfate conditions with enhanced desulfonation may potentially be used to enhance crop yields (Kertesz & Mirleau, 2004), but they may also be important in other ways. For example, it has been shown that potato products processed from tubers grown with low sulfate fertilizer contain four-fold lower levels of the carcinogen acrylamide than when grown with excess sulfate (Elmore *et al.*, 2007) and the presence of desulfonating strains might be useful to enhance the tolerance of crop plants to low sulfate conditions. The results presented here, however, suggest that at least for rhodococci the natural population could be insufficient for this purpose, and additional inoculation with suitable isolates will be required to ensure a robust response to low-sulfate conditions. Survival and activity of these strains under a range of soil conditions needs to be examined in detail in future studies.

Acknowledgements

We thank Sarah Hodge and Harriet Denton for their technical assistance, Adam Huffman for bioinformatics assistance and Linday Eltis for the providing the

Rhodococcus RHA1 strain. This work was supported by the Natural Environment Research Council (NERC). Rothamsted Research receives grant-aided support from the Biotechnology and Biological Science Research Council (BBSRC) of the UK.

References

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**:403-410.

Autry AR & Fitzgerald JW (1990) Sulfonate S - A major form of forest soil organic sulfur. *Biol. Fertil. Soils* **10**:50-56.

Beil S, Kehrli H, James P, Staudenmann W, Cook AM, Leisinger T & Kertesz MA (1995) Purification and characterization of the arylsulfatase synthesized by *Pseudomonas aeruginosa* PAO during growth in sulfate-free medium and cloning of the arylsulfatase gene (*atsA*). *Eur. J. Biochem.* **229**:385-394.

Böhme L, Langer U & Böhme F (2005) Microbial biomass, enzyme activities and microbial community structure in two European long-term field experiments. *Agric. Ecosyst. Environ.* **109**:141-152.

Clegg CD, Lovell RDL & Hobbs PJ (2003) The impact of grassland management regime on the community structure of selected bacterial groups in soils. *FEMS Microbiol. Ecol.* **43**:263-270.

Cunliffe M & Kertesz MA (2006) Effect of *Sphingobium yanoikuyae* B1 inoculation on bacterial community dynamics and polycyclic aromatic hydrocarbon degradation in aged and freshly PAH-contaminated soils. *Environ. Pollut.* **144**:228-237.

Curl E & Truelove B (1986) *The rhizosphere* Springer-Verlag, Heidelberg.

1 Denome SA, Olsen ES & Young KD (1993) Identification and cloning of genes
2 involved in specific desulfurization of dibenzothiophene by *Rhodococcus* sp.
3 strain IGTS8. *Appl. Environ. Microbiol.* **59**:2837-2843.
4 Drinkwater LE & Snapp SS (2007) Understanding and managing the rhizosphere in
5 agroecosystems. The Rhizosphere. An ecological perspective, (ZG Cardon &
6 JL Whitbeck, eds), p. 127-154. Elsevier, Amsterdam.
7 Elmore JS, Mottram DS, Muttucumaru N, Dodson AT, Parry MAJ & Halford NG
8 (2007) Changes in free amino acids and sugars in potatoes due to sulfate
9 fertilization and the effect on acrylamide formation. *J. Agric. Food Chem.*
10 **55**:5363-5366.
11 Enwall K, Nyberg K, Bertilsson S, Cederlund H, Stenstrom J & Hallin S (2007)
12 Long-term impact of fertilization on activity and composition of bacterial
13 communities and metabolic guilds in agricultural soil. *Soil Biol. Biochem.*
14 **39**:106-115.
15 Freeman C & Nevison GB (1999) Simultaneous analysis of multiple enzymes in
16 environmental samples using methylumbelliferyl substrates and HPLC. *J.*
17 *Environ. Qual.* **28**:1378-1380.
18 Gallagher JR, Olson ES & Stanley DC (1993) Microbial desulfurization of
19 dibenzothiophene - a sulfur-specific pathway. *FEMS Microbiol. Lett.* **107**:31-
20 36.
21 Gardener BBM & Weller DM (2001) Changes in populations of rhizosphere bacteria
22 associated with take-all disease of wheat. *Appl. Environ. Microbiol.* **67**:4414-
23 4425.

1 Germida JJ & Siciliano SD (2001) Taxonomic diversity of bacteria associated with
2 the roots of modern, recent and ancient wheat cultivars. *Biol. Fertil. Soils*
3 **33**:410-415.
4 Ghani A, McLaren RG & Swift RS (1992) Sulphur mineralisation and
5 transformations in soils as influenced by additions of carbon, nitrogen and
6 sulphur. *Soil Biol. Biochem.* **24**:331-341.
7 Gilbert SC, Morton J, Buchanan S, Oldfield C & McRoberts A (1998) Isolation of a
8 unique benzothiophene-desulphurizing bacterium, *Gordona* sp. strain 213E
9 (NCIMB 40816), and characterization of the desulphurization pathway.
10 *Microbiology* **9**:2545-2553.
11 Goodfellow M (1971) Numerical taxonomy of some nocardioform bacteria. *J. Gen.*
12 *Microbiol.* **69**:33-80.
13 Gupta N, Roychoudhury PK & Deb JK (2005) Biotechnology of desulfurization of
14 diesel: prospects and challenges. **66**:356-366.
15 Hartmann M & Widmer F (2006) Community structure analyses are more sensitive to
16 differences in soil bacterial communities than anonymous diversity indices.
17 *Appl. Environ. Microbiol.* **72**:7804-7812.
18 Heuer H, Krsek M, Baker P, Smalla K & Wellington EMH (1997) Analysis of
19 actinomycete communities by specific amplification of genes encoding 16S
20 rRNA and gel-electrophoretic separation in denaturing gradients. *Appl.*
21 *Environ. Microbiol.* **63**:3233-3241.
22 Hinsinger P, Gobran GR, Gregory PJ & Wenzel WW (2005) Rhizosphere geometry
23 and heterogeneity arising from root-mediated physical and chemical processes.
24 *New Phytol.* **168**:293-303.

1 Hirasawa K, Ishii Y, Kobayashi M, Koizumi K & Maruhashi K (2001) Improvement
2 of desulfurization activity in *Rhodococcus erythropolis* KA2-5-1 by genetic
3 engineering. *Biosci. Biotechnol. Biochem.* **65**:239-246.
4 Innerebner G, Knapp B, Vasara T, Romantschuk M & Insam H (2006) Traceability of
5 ammonia-oxidizing bacteria in compost-treated soils. *Soil Biol. Biochem.*
6 **38**:1092-1100.
7 Kertesz MA & Wietek C (2001) Desulfurization and desulfonation. Sulfur-controlled
8 gene expression in bacteria. *Appl. Microbiol. Biotechnol.* **57**:460-466.
9 Kertesz MA & Mirleau P (2004) The role of soil microbes in plant sulfur nutrition. *J.*
10 *Exp. Bot.* **55**:1939-1945.
11 Kertesz MA, Fellows E & Schmalenberger A (2007) Rhizobacteria and plant sulfur
12 supply. *Adv. Appl. Microbiol.* **62**:235-268.
13 Kilbane JJ (2006) Microbial biocatalyst developments to upgrade fossil fuels. *Curr.*
14 *Opin. Biotechnol.* **17**:305-314.
15 Kilbane JJ & Jackowski K (1992) Biodesulfurization of water-soluble coal-derived
16 material by *Rhodococcus rhodocrous* IGTS8. *Biotechnol. Bioeng.* **40**:1107-
17 1114.
18 Klose S, Moore JM & Tabatabai MA (1999) Arylsulfatase activity of microbial
19 biomass in soils as affected by cropping systems. *Biol. Fertil. Soils* **29**:46-54.
20 Knauff U, Schulz M & Scherer HW (2003) Arylsufatase activity in the rhizosphere
21 and roots of different crop species. *Eur. J. Agron.* **19**:215-223.
22 Landa BB, Mavrodi OV, Schroeder KL, Allende-Molar R & Weller DM (2006)
23 Enrichment and genotypic diversity of phlD-containing fluorescent
24 *Pseudomonas* spp. in two soils after a century of wheat and flax monoculture.
25 *FEMS Microbiol. Ecol.* **55**:351-368.

1 Lane DJ (1991) 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial
2 systematics, (E Stackebrandt & M Goodfellow, eds), p. 115-147. Wiley, New
3 York.
4 Larkin MJ, Kulakov LA & Allen CCR (2005) Biodegradation and *Rhodococcus* -
5 masters of catabolic versatility. *Curr. Opin. Biotechnol.* **16**:282-290.
6 Maghsoudi S, Kheirolomoom A, Vossoughi M, Tanaka E & Katoh S (2000) Selective
7 desulfurization of dibenzothiophene by newly isolated *Corynebacterium* sp.
8 strain P32C1. *Biochem. Eng. J.* **5**:11-16.
9 Maghsoudi S, Vossoughi M, Kheirolomoom A, Tanaka E & Katoh S (2001)
10 Biodesulfurization of hydrocarbons and diesel fuels by *Rhodococcus* sp. strain
11 P32C1. *Biochem. Eng. J.* **8**:151-156.
12 Marschner P, Kandeler E & Marschner B (2003) Structure and function of the soil
13 microbial community in a long-term fertilizer experiment. *Soil Biol. Biochem.*
14 **35**:453-461.
15 Mazzola M, Funnell DL & Raaijmakers JM (2004) Wheat cultivar-specific selection
16 of 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* species
17 from resident soil populations. *Microb. Ecol.* **48**:338-348.
18 McGrath SP & Zhao FJ (1995) A risk assessment of sulfur deficiency in cereals using
19 soil and atmospheric deposition data. *Soil Use Manage.* **11**:110-114.
20 McLeod MP, Warren RL, Hsiao WWL*, et al.* (2006) The complete genome of
21 *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. *Proc.*
22 *Natl. Acad. Sci. U. S. A.* **103**:15582-15587.
23 Meyer AJ & Hell R (2005) Glutathione homeostasis and redox-regulation by
24 sulfhydryl groups. *Photosynth. Res.* **86**:435-457.

1 Muyzer G, De Waal ED & Uitterlinden AG (1993) Profiling of complex microbial
2 populations by denaturing gradient gel electrophoresis analysis of polymerase
3 chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ.*
4 *Microbiol.* **59**:695-700.
5 Oldfield C, Pogrebinsky O, Simmonds J, Olson ES & Kulpa CF (1997) Elucidation of
6 the metabolic pathway for dibenzothiophene desulphurization by *Rhodococcus*
7 sp. strain IGTS8 (ATCC 53968). *Microbiology* **9**:2961-2973.
8 Pearson WR & Lipman DJ (1988) Improved tools for biological sequence
9 comparison. *Proc. Natl Acad. Sci. USA* **85**:2444-2448.
10 Rothamsted_Research (2006). Guide to the Classical and other Long-term
11 Experiments, Datasets and Sample Archive:
12 http://www.rothamsted.bbsrc.ac.uk/resources/LongTermExperiments.pdf.
13 *Accessed 1 February 2008.*
14 Rothamsted_Research (2008) *Electronic Rothamsted Archive:*
15 *http://www.rothamsted.bbsrc.ac.uk/eRA/. Accessed 1st February, 2008.*
16 Scherer HW (2001) Sulphur in crop production. *Eur. J. Agron.* **14**:81-111.
17 Schleheck D, Lechner M, Schonenberger R, Suter MJF & Cook AM (2003)
18 Desulfonation and degradation of the disulfodiphenylethercarboxylates from
19 linear alkyldiphenyletherdisulfonate surfactants. *Appl. Environ. Microbiol.*
20 **69**:938-944.
21 Schmalenberger A & Tebbe CC (2002) Bacterial community composition in the
22 rhizosphere of a transgenic, herbicide-resistant maize (*Zea mays*) and
23 comparison to its non-transgenic cultivar Bosphore. *FEMS Microbiol. Ecol.*
24 **40**:29-37.

Schmalenberger A & Kertesz MA (2007) Desulfurization of aromatic sulfonates by rhizosphere bacteria - high diversity of the *asfA* gene. *Environ. Microbiol.* **9**:535-545.

Schmalenberger A, Schwieger F & Tebbe CC (2001) Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. *Appl. Environ. Microbiol.* **67**:3557-3563.

Schmalenberger A, Drake HL & Küsel K (2007) High unique diversity of sulfate-reducing prokaryotes characterized in a depth gradient in an acidic fen. *Environ. Microbiol.* **9**:1317-1328.

Schmalenberger A, Hodge S, Bryant A, Hawkesford MJ, Singh BK & Kertesz MA (2008) The role of *Variovorax* and other Comamonadaceae in sulfur transformations by microbial wheat rhizosphere communities exposed to different sulfur fertilization regimes. *Environ. Microbiol.* **10**:in press.

Seghers D, Siciliano SD, Top EM & Verstraete W (2005) Combined effect of fertilizer and herbicide applications on the abundance, community structure and performance of the soil methanotrophic community. *Soil Biol. Biochem.* **37**:187-193.

Seghers D, Top EM, Reheul D, Bulcke R, Boeckx P, Verstraete W & Siciliano SD (2003) Long-term effects of mineral versus organic fertilizers on activity and structure of the methanotrophic community in agricultural soils. *Environ. Microbiol.* **5**:867-877.

Sharma SS & Dietz KJ (2006) The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J. Exp. Bot.* **57**:711-726.

1 Sikkema J & Debont JAM (1993) Metabolism of tetralin (1,2,3,4-
2 tetrahydronaphthalene) in *Corynebacterium* sp. strain C125. *Appl. Env.*
3 *Microbiol.* **59**:567-572.
4 Stark C, Condron LM, Stewart A, Di HJ & O'Callaghan M (2007) Influence of
5 organic and mineral amendments on microbial soil properties and processes.
6 *Appl. Soil Ecol.* **35**:79-93.
7 Taylor JP, Wilson B, Mills MS & Burns RG (2002) Comparison of microbial
8 numbers and enzymatic activities in surface soils and subsoils using various
9 techniques. *Soil Biol. Biochem.* **34**:387-401.
10 van der Geize R & Dijkhuizen L (2004) Harnessing the catabolic diversity of
11 rhodococci for environmental and biotechnological applications. *Curr. Opin.*
12 *Microbiol.* **7**:255-261.
13 Vermeij P, Wietek C, Kahnert A, Wüest T & Kertesz MA (1999) Genetic
14 organization of sulfur-controlled aryl desulfonation in *Pseudomonas putida* S-
15 313. *Mol. Microbiol.* **32**:913-926.
16 Vong PC, Dedourge O, Lasserre-Joulin F & Guckert A (2003) Immobilized-S,
17 microbial biomass-S and soil arylsulfatase activity in the rhizosphere soil of
18 rape and barley as affected by labile substrate C and N additions. *Soil Biol.*
19 *Biochem.* **35**:1651-1661.
20 Zhao FJ, Wu J & McGrath SP (1996) Soil organic sulphur and its turnover. Humic
21 substances in terrestrial ecosystems, (A Piccolo, eds), p. 467-506. Elsvier,
22 Amsterdam.
23 Zhao FJ, Knights JS, Hu ZY & McGrath SP (2003) Stable sulfur isotope ratio
24 indicates long-term changes in sulfur deposition in the Broadbalk experiment
25 since 1845. *J. Environ. Qual.* **32**:33-39.

Zhao FJ, Lehmann J, Solomon D, Fox MA & McGrath SP (2006) Sulphur speciation and turnover in soils: evidence from sulphur K-edge XANES spectroscopy and isotope dilution studies. *Soil Biol. Biochem.* **38**:1000-1007.

Zürrer D, Cook AM & Leisinger T (1987) Microbial desulfonation of substituted naphthalenesulfonic acids and benzenesulfonic acids. *Appl. Environ. Microbiol.* **53**:1459-1463.

Table 1. Bacterial strains and oligonucleotides used in this study

Strain or primer	Description	Source
Bacteria		
Rhodococcus erythropolis IGTS8	Dibenzothiophene-utilizing isolate	(Kilbane & Jackowski, 1992)
Rhodococcus erythropolis NCIMB 11148	Type strain	(Goodfellow, 1971)
Rhodococcus opacus DSM 8531	Toluene-utilizing isolate	(Sikkema & Debont, 1993)
Rhodococcus sp. RHA1	Polychlorinated biphenyl degrader	(McLeod *et al.*, 2006)
Rhodococcus sp. P14D10	Toluenesulfonate-utilizing isolate	This study
Rhodococcus sp. P15D9	Toluenesulfonate-utilizing isolate	This study
Oligonucleotides		
1492r	TACGGTTACCTTGTTACGACTT	(Lane, 1991)
27f	AGAGTTTGATCCTGGCTCAG	(Lane, 1991)
518R	ATTACCGCGGCTGCTGG	(Muyzer *et al.*, 1993)
GC-341F	CGCCCGCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGGGGCCTACGGGAGGC AGCAG	(Muyzer *et al.*, 1993)
asfBtoA	ASCTCGCACATGAAGCAGGT	(Schmalenberger & Kertesz, 2007)
F243Actino	GGATGAGCCCGCGGCCTA	(Heuer *et al.*, 1997)

asfA_F_rho1	AATGAGACTGGGGCAACCCAA	This study
asfA_rho_QF	ARTTCTCCAACGCGTACG	This study
asfA_rho_QR	TCGAATTGCAGGAAGAAGTTG	This study
asfA_RHA1	GACGTCGTCCTCGTAGACAA	This study

1

2

3

Table 2. Growth of *Rhodococcus* strains P14D10 and P15D9 in minimal medium with a range of sulfur sources.[a]

Sulfur source	***Rhodococcus* sp. P14D10**	***Rhodococcus* sp. P15D9**
Arylsulfonates:		
o-Aminobenzenesulfonate	++	++
4-Nitrobenzenesulfonate	++	++
Naphthalene-2-sulfonate	++	++
Toluenesulfonate	++	++
Benzenesulfonate	++	++
Alkanesulfonates:		
Methanesulfonate	++	++
Pentanesulfonate	++	++
Dodecanesulfonate	++	++
3-Morpholinopropanesulfonate	++	++
Taurine	++	++
Cysteate	++	++
Sulfate esters:		
Methylsulfate	++	++
Nitrocatecholsulfate	++	++
Sodium dodecylsulfate	-	+
4-Nitrophenolsulfate	++	++
Amino acids:		
Glutathione	++	++
Cysteine	++	++
Homocysteine	++	++
Methionine	++	++

Other sulfur sources:		
Sodium sulfate	++	++
Potassium thiocyanate	-	+
Dimethylsulfone	++	++
Dibenzothiophene	-	-
Dimethylsulfoxide	++	++

a) Cells were grown aerobically in minimal medium with 250 μM sulfur source. Growth was monitored as optical density at stationary phase: ++, growth to an OD_{600} above 0.8; +, significant growth below an OD_{600} of 0.8; -, no growth compared to the sulfur-free control.

Table 3. Expression of *asfA* homologues in *Rhodococcus* strains P14D10, P15D9 and RHA1 (copies of *asfA* RNA per ng of total RNA) during growth in minimal medium with different sulfur sources.

Sulfur source (250 μM)	*Rhodococcus* sp. P14D10	*Rhodococcus* sp. P15D9	*Rhodococcus* sp. RHA1
TS	$5.35 \pm 0.61 \times 10^5$	$4.12 \pm 0.74 \times 10^5$	$3.17 \pm 0.10 \times 10^4$
TS+Sulfate	$1.07 \pm 0.06 \times 10^3$	$1.25 \pm 0.08 \times 10^3$	$0.95 \pm 0.06 \times 10^3$
Sulfate	$1.15 \pm 0.04 \times 10^3$	$0.9 \pm 0.08 \times 10^3$	$0.44 \pm 0.01 \times 10^3$

a). Expression was evaluated by quantitative RT-PCR as described in Experimental procedures. TS: toluenesulfonate.

Fig. Legends

Fig. 1. Characterization of the actinobacterial community amplified from the rhizosphere of winter wheat at the Broadbalk long-term experiment. (a) Denaturing gradient electrophoresis of 16S rRNA gene fragments from the following fertilization regimes. NIL – no fertilizer application; NPK – inorganic fertilizer without sulfur; NPKS – inorganic fertilizer with sulfate; FYM – farmyard manure. Replicates 1 and 2 originated from section 9 and replicates 3 and 4 originated from section 1 of the Broadbalk experiment. M - Marker lane containing selected 16S rRNA genefragments with appropriate mobility. Sequence information from selected signals was obtained by cloning and sequencing or via direct sequencing (band numbers underlined). (b). Dendrogram representing UPGMA analysis of the DGGE profiles using Phoretix software.

Fig. 2. Tree of partial N-terminally truncated protein sequences of the oxidoreductase AsfA and its orthologues, accomplished by using a maximum likelihood (Dayhoff model) method. Sequences retrieved from this study are the molecular isolates from the clone library of wheat rhizosphere (RhoC1-RhoC70) and the isolates from the wheat rhizosphere (bold). The AsfA sequences from reference organisms such as *Variovorax paradoxus* was retrieved from an earlier study (Schmalenberger & Kertesz, 2007) and from GenBank respectively.

Fig. 3. Ordination plot of multiplex RFLP/T-RFLP profiles of *asfAB* amplified from the rhizosphere of winter wheat from the Broadbalk long-term experiment, subjected to different fertilization regimes: NIL (squares), NPK (circles), NPKS (triangles) and

1 FYM (diamonds). RFLP and T-RFLP analysis were carried out as described in
2 Experimental Procedures. Detrended correspondence analysis (DCA) was performed
3 using Decorana software. Details of the individual treatments are given in the legend
4 to Fig. 1
5
6